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IMMUNOLOGICAL APPROACH IN SCHISTOSOMIASIS.(U)
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Immunological Approach in Schistosomiasis.

Dr. Fatma/Hassan

Assisted By : S. Sedlik, A. Shetta and A. Nousseir

Annual Report

Contract No. / W 0014 - 75 - C - 0835

The following items were the main subjects of our study during the last year .

Study No. 1 with reference to our last report of 1978 immunization experiments with high doses of irradiation with Cobalt 60 are terminated and the study with its results are as follows .

In a previous study a trial of inducing protection by Immunization of mice with low doses of cobalt . 60 irradiated cercariae was carried out .

The results were rather encouraging yet follow up of immunized animals failed to give satisfactory resistance to new infection .

However protection to Schistosoma infection with attenuated parasite has been reported in several animal models using/ irradiated cercariae by ultra violet ,
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x or γ rays . Single or multiple doses were found to give different end results in the over all protection.

The present study deals with induction of protective immunity by using high doses of irradiation in attenuating

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the cercariae . The study deals also with investigating the mechanism of immunity by evaluating the changes in humoral , cell mediated and immunopathological variations.

Mice used in all experiments were inbred 6 weeks old female (Charles River) strain .

Cercariae : -

Pooled cercariae from large numbers of Planorbis boissyi snails infected with an Egyptian strain of S.mansoni .

Cobalt 60 Irradiation :-

Central uniform irradiation was applied from a Gamma cell 220 cobalt 60 " Noratom Nor Control" at the National Institute of Radiology Dokki Center. This cell delivered 90 rads/second . Cercariae were suspended in about 50 ml of dechlorinated tap water . Irradiated cercariae were used within 30 minutes of irradiation.

Infection :-

All cercariae whether irradiated or non irradiated were administered , percutaneously by tail immersion.

Recovery of adult worms :-

Adult worms recovered from mesenteric veins by perfusion , 8 weeks following the infection .

Calculation : -

Resistance is expressed according to Method of Minard¹⁵
et al²⁰ as percent reduction in number of worms recovered from experimental animals .

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Where Ic = immunized and challenged animals

C = non immunized and challenged only.

I = immunized and non challenged

Thus $\frac{I-(Ic-I) \times 100}{C}$ = % reaction for individual mouse.

EAC rosette forming lymphocytes :-

B- lymphocyte cell population was prepared from the spleens of newly killed mice. Spleen cells were separated by dissection then were suspended in phosphate buffered saline(PBS) of pH 7.2 . These cells were then washed 5 times by centrifugation at 2000 rpm for 5 minutes in the same medium . The washed cells were then suspended in minimum essential Medium (MEM) supplemented with 15% foetal calf serum , and diluted in such a way that the cell count/ml will amount to 4×10^6 .

Preparation of sheep erythrocytes(E) :-

Sheep erythrocytes (E) were sensitized with anti-sheep erythrocytes (A) and mice complement (c) by the modified method of Shevach et al . Equal volumes of sheep erythrocytes of 0.5 % were collected in equal volumes of sterile Alzvers solution and stored at $+4^{\circ}C$ then used after 48 hours.(E) cells were washed 3 times with PBS then 0.5 ml was taken and sensitized with a subagglutinating dilution of rabbit anti SRBC hemolysen incubate for 30 minutes then centrifuge and wash . The washed(EA) was further incubated with 1/10 dilution of fresh frozen mouse complement (EAC) then is resuspended in (MEM) + 15% foetal calf serum 0.2 of this latter preparation is added to 0.2 ml of the previously prepared lymphocyte solution . Mixture is centrifuged at 20xg for 5 minutes then incubated for 30 minutes . Cells were then vigorously resuspended and percentages of rosette forming cells were calculated .

Antibody estimation :-

Elisa technique was used in evaluating the circulating antibody directed against schistosoma mansoni adult worm antigen. 1ml of (6 ug/ml of semipurified antigen is dissolved in 0.02% sodium azide). This was incubated at 37 °C in polystyrene tubes for 3 hours, then kept at + 4 °C till they are used. Just before using the tubes are washed 3 times with (P.B.S.) containing 0.05% tween 20, 1ml of the appropriately diluted mice sera (1/100) with P.B.S are incubated for 6 hours at room temperature, excess serum is removed and washed as before. 1ml of diluted antiserum (0.5ug/ml) marked with peroxidase is added and incubated overnight. Excess of conjugate is removed. The utilized substrate (hydrogen peroxide + orthodianisidine) is put in contact with the fixed enzyme for 1 hour. The developed yellow colour is measured in a spectrophotometer at 400 mu. The optimal density given will be finally proportional to the concentration of antibody of the infected mice sera Voller et al.¹⁷

Immunopathological Study :-

Methods of Staining :

Two stains are used ; haematoxylin and eosin stain and the Dominici's stain specific for eosinophils used by Colley et al.¹⁸.

Results & Discussion:

Results of worm load, rosette forming lymphocytes, Elisa and percentage of animals developing liver are shown in Table(1).

From our results, mice immunized with 100 n.cercariae were found to acquire detectable resistance to percutaneous cercarial challenge as evidenced by 77.8% reduction in worm load. This is in accordance to results reported by Sher et al. In groups receiving irradiated cercariae, maximum reduction in worm load

after challenge was that observed in the groups receiving 20 Kr. units (88.55%). This is probably due to the immunization effect of the immature stages of the parasite as irradiation dose above 20 Kr. prevent cercariae from reaching maturity Vilella and Weinborn²⁰. Also Philips et al 1977²¹ reported that the immunologically mediated resistance is most effectively stimulated by immature stages of infection.

The percentage of EAC rosette forming lymphocytes in normal infection was slightly increased, while a marked depression in those receiving the irradiated cercariae was observed. This might be due to the depression of B lymphocytes caused by the effect of the penetrating irradiated antigen. The non immunized infected control age group showed the highest percentage of EAC formation which could be explained by the full maturation of the immune system of the animal at that age. All groups on challenge could not recover from that depression except that receiving 20 Kr. dose, which showed a remarkable increase in the antibody forming cells.

On the otherhand specific antischistosomal antibody detected by the Elisa titres showed least values in the 40Kr. group, moderate in the normal control and highest in the rest of the irradiated groups.

The decrease in the specific antibody titre observed with the 20 Kr. group may be due to the utilization of this antibody in the different antibody dependent killing mechanisms reported by many workers Sher et al²², Dean et al²³, Perez²⁴, Capron et al²⁵, Butterworth et al^{26,27,28}. The specific antibody in all other groups is somewhat increased in the peripheral blood as it is not incorporated in killing

mechanism probably due to immature membrane of effector cells . Also it might not be involved in immunocomplex formation . More work is in advance to test the cytotoxic effect of this antibody obtained at normal and different irradiation doses of infection .

However the 20 Kr. dose was found to be the most effective irradiating dose that gives the maximum protection in our dose range used . Moreover there may be lots of other factors that would affect immunologically the protection mechanism .

In conclusion from our results we could say that there is no direct correlation between protection and antibody levels .

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Table: Mean values, of worm load, % of rosette forming lymphocytes, ELISA and liver histopathology

Group	Dose of irradiation (rads)	Worm Load		% of Worm reduction	EAC		Antibody Levels (ELISA)		% of animals with liver granulomata .	
		Before Challenge	After Challenge		Before Challenge	After Challenge	Before Challenge	After Challenge	Before Challenge	After Challenge
oo 1	Normal Inf.	29.6+2.0	5.95+7.61	77.8	9.0+1.1	9.5+1.96	0.430+0.02	0.990+0.09	100	100
ll	Control	-	26.6 +5.07	-	-	11.3+3.5	-	0.712+0.07	-	100
lll	10,000	0	13.8 +4.39	48.23	9.0+0	3.1+1.29	0.528+0.01	0.575+0.04	0	53.33
IV	20,000	0	4.00+2.26	84.56	2.4+1.2	10.0+1.41	0.40+0.08	0.655+0.15	0	92.53
V	30,000	0	7.16+3.89	73.08	1.0+0	1.0	0.413+0.03	0.621+0.04	0	52.53
VI	40,000	0	5.8+1.22	78.19	1.0+0	2.6+0.47	0.424+0.18	0.776+0.11	0	60.03
VII	50,000	0	5.5 +2.79	79.32	2.6+0.47	3.66+2.08	0.504+0.06	0.751+0.1	0	59.23
ooo VIII	60,000	0	6.1 +7.1	77.06	2.23+0.47	1.65+0.47	0.411+0.0	0.750+1.1	0	4.53
IX	Normal control age	-	-	-	4.3+1.5	4.8+2.1	0.401+0.15	0.430+0.12	-	-

oo Control non infected but challenged only .

ooo Control age group non infected and non challenged .

Study No. 11

After standardization of the Eliza technique in our lab., it was necessary to evaluate its results and compare it with other routine techniques.

In a previous paper, Hassan et al 1978 studied 34 bilharzial patients divided into 5 clinical groups. Quantitative immunoglobulin estimations, qualitative immunodiffusion of antigen and antibody titres, immunoelectrophoresis (IEP), indirect haemagglutination (IHA) test and cell mediated immunity tests were performed for all the patients. IHA was found to be the most sensitive test (positivity rate 70.5 %) comparatively followed by IEP (58.8 %) and immunodiffusion (55.9 %).

Enzyme linked immunosorbent assay - (ELISA) first described by Engvall and Perlman 1972 was used to detect antibodies to *Trichinella spiralis* Nuitenberget al 1973. *Trypanosoma cruzi* Voller et al 1975, Malaria parasites Voller et al 1975b, *Toxoplasma gondii* Voller 1976. Hydatidosis, Bout 1975 and Amoebiasis Bos and Euk 1975, Hulit et al 1975 found that ELISA can be used to distinguish between *Schistosoma* infected and uninfected individuals, even in cases where conventional serological methods such as indirect fluorescent antibody (IFA) tests fail to detect antibodies.

The largest survey using ELISA to date is that of Melanin et al 1978 who found it very efficient in detecting infected people in populations where the disease is endemic.

The aim of the present work is to compare and evaluate the efficiency of three highly sensitive techniques for measuring specific antischistosome antibodies namely : ELISA, IFA and IHA tests .

Patients :-

Eighty six patients with active bilharzial infection were studied . All were males . Age ranged between 8-60 years . They were classified into 5 clinical groups . Group I : Forty eight early urinary bilharzial patients . They had a history of bilharzial infection of less than 3 years; their main complaint was haematuria and / or dysuria . Clinical examination revealed neither enlargement of the liver or the spleen . Group II : Nine late urinary bilharzial patients . Symptoms and signs were similar to group I . They all had calcification of the bladder and / or the ureters . Group III : four early intestinal bilharzial patients . They complained of dysentery or diarrhoea . Clinical examination revealed no enlargement of the liver or the spleen . Group IV : Eighteen hepatosplenomegalic cases . Their main complaint was abdominal discomfort after meals , diarrhoea and / or dysentery . They all had enlargement of the liver and the spleen . Group V : Seven mixed bilharzial cases . Their main complaint was haematuria and / or dysuria and abdominal discomfort . They all had enlargement of the liver and the spleen . Group I , II and V passed viable *S.haematobium* ova in the urine . Group III and IV had viable *S.mansoni* ova in stools . Group V had viable *S.mansoni* ova in rectal snips .

Twelve normal individuals with no history of bilharzial infection and with negative intradermal and circumoval precipitin tests for bilharziasis were considered as controls.

Patients harbouring any parasite other than bilharziasis were excluded from the study to eliminate cross reactions with other helminthic infections Schinski et al 1976 .

The 3 major adopted techniques used in our study were ELISA, IFA and IHA .

Enzyme linked immunosorbent assay :-

ELISA technique used can be described in the following: A semipurified worm antigen is dissolved in 0.01 M phosphate buffer solution (PBS) of pH 7.2 containing 0.02 percent sodium azide . 2ml of antigen solution (6 ug / ml) was incubated at 37 °C in polystyrene tubes(Biomat Hazebrouck) for 3 hours , then kept at + 4 °C till they are used. Just before using , the tubes are washed 3 times with PBS containing 0.05 percent of tween 20 then the tubes are evacuated by aspiration and washed 3 times . 2ml of the appropriately diluted serum (1/500) with above PBS are incubated for 6 hours at room temperature , excess of serum is removed and washed as before . 2ml of the diluted conjugate(peroxidase labelled antihuman immunoglobulin) is added and incubated for 1 hour. Excess of conjugate is removed . The used substrate (hydrogen peroxide plus orthodianisidine) is put in contact with the fixed enzyme for 1 hour . The developed yellow colour is measured in a spectrophotometer at 400 nm . The

optimal density given will be proportional to the concentration of antibody in the patient's serum . Engvall and Perlman 1972 .

Indirect fluorescent antibody test:-

IFA technique was carried out by using frozen sections of adult worms as recommended by Wilson et al 1974. Formalin fixed whole worm antigen with a fluorescein isothiocyanate labelled rabbit antihuman IgG (Pasteur) at a dilution of 1/50-1/100 is used .

Indirect haemagglutination test :-

The microtitre IHA technique for detection of antibodies was carried out by using 10mg worm antigen . Starting dilution was 1/80 and the final one 1/5120 Hassan et al 1978.

All the results were verified at Immunological Parasitology Department . Medical College , Lille , under the supervision of Professor A. Capron using the same technique and practically the same reagents .

Results and Discussion : -

Results are shown in tables I, II and III.

Specific antischistosome antibodies are a major factor in the development of acquired resistance to bilharziasis Sher et al 1975 . There is a great need for highly sensitive assays to measure accurately the amount of these antibodies as current procedures are relatively insensitive. Fife 1971.

The mean values of ELISA, IFA, IHA were significantly higher in all our groups of patients as compared to controls . The humoral antibodies detected by either methods (ELISA, IFA,

and IHA) showed significant increasing values with the progress of the disease which is parallel to the increase in the antigenicity of the disease Hassan et al 1978.

The positivity rate of ELISA in our bilharzial patients was 82.6 percent and 80.2 percent (in Egypt and Lille respectively) , of IFA 79.1 percent and of IHA 77.9 percent and 75.6 percent (in Egypt and Lille respectively). ELISA gave the most sensitive results . The failure of ELISA to detect antibodies in 17.4 percent and 19.8 percent (in Egypt and Lille respectively) of our bilharzial patients with active infection may be explained by the antigen binding of antibodies forming immune complexes Hassan et al 1978.

Both the positivity rate and the mean values of antibody titre recorded by the 3 diagnostic techniques under evaluation either in Egypt or Lille were significantly higher in mansoni than haematobium infections . This may be explained by species specificity of antibody response since the used antigen was always that of mansoni species . This finding agrees with the results concerning ELISA reported by memorandum issued by WHO in 1976 , Schinski et al 1976 could not find any statistically significant difference between the antibody titres detected from sera of patients with the 2 species of infection using IHA technique .

Comparative data of IHA in Egypt and Lille showed no significant difference . On the other hand , a slight significant increase in values in Egypt compared to Lille was recorded when using the ELISA technique . This may be due to the fact that the base line of the normal individuals in Egypt

is significantly higher than that of Lille . This may be explained by the normal high gamma globulin content mostly recorded in the Egyptians subsequent to parasitic & bacterial infections Makled 1972.

The superiority of the ELISA proved by our results might be due to the ability of one enzyme molecule to react with numerous molecules of substrate , thereby amplifying the sensitivity of detection of the enzyme , moreover ELISA has many advantages ; minute quantities of serum or plasma are needed ; a simple portable spectrophotometer can be used under field conditions ; qualitative results can be detected by the naked eye ; shelf life of reagents is rather long and health hazards for laboratory personnel are practically none . Voller et al 1977 showed that ELISA was just as sensitive as radioimmunoassay for schistosomal antibody yet the above mentioned advantages mean that ELISA is more convenient for use in most laboratories .

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Table I : Positivity Rate of ELISA, IHA and TMA Tests

Group	No.	Age range years	Egypt	Lille	IHA	ELISA	Lille
I: Early urinary	48	10 - 45	36 (75%)	34 (70.8%)	32 (66.6%)	31 (64.5%)	29 (60.4%)
II: Late urinary	09	12 - 26	08 (88%)	08 (88%)	09 (100%)	09 (100%)	09 (100%)
III: Early intestinal	04	10 - 22	03 (75%)	03 (75%)	04 (100%)	03 (75%)	03 (75%)
IV: Hepato-spleno-megaly	18	8 - 60	17 (94.4%)	17 (94.4%)	16 (88.8%)	17 (94.4%)	17 (94.4%)
V: Mixed	07	14 - 26	07 (100%)	07 (100%)	07 (100%)	07 (100%)	07 (100%)
Total	86	8 - 60	71 (82.6%)	69 (80.2%)	68 (79.1%)	67 (77.9%)	65 (75.6%)

Table II : Mean values of ELISA, IHA and IHA Results

Group	No.	ELISA		IHA	IHA	
		Egypt	Lille	Egypt	Lille	
I : Early urinary	6	0.252 ± 0.175	0.223 ± 0.113	1/2 ± 1/15	1/239 ± 1/401	1/112 ± 1/287
II : Late urinary	29	0.257 ± 0.142	0.377 ± 0.210	1/31 ± 1/427	1/2915 ± 1/1734	1/2447 ± 1/1713
III : Early intestinal	26	0.430 ± 0.239	0.414 ± 0.201	1/150 ± 1/423	1/210 ± 1/273	1/200 ± 1/153
IV : Mucato - Splenoregally	13	0.602 ± 0.223	0.536 ± 0.223	1/155.9 ± 1/444.5	1/1715 ± 1/1574	1/1451 ± 1/1516
V : Mixed	27	0.704 ± 0.100	0.609 ± 0.213	1/265 ± 1/403	1/2100 ± 1/2209	1/2154 ± 1/1561
Total	86	0.524 ± 0.239	0.433 ± 0.253	1/247 ± 1/333	1/1079 ± 1/1510	1/358 ± 1/1212
Control	12	0.192 ± 0.046	0.131 ± 0.039	Negative	Negative	Negative

- Values are slightly (*), moderately (**), highly (***) and very highly (****) significant as compared to controls
- P value < 0.005 is slightly, < 0.0025 is moderately, < 0.0025 is highly and < 0.0005 is highly significant values.
- No significant difference was detected between the total mean IHA in Egypt and Lille ($p > 0.05$) but there was slight significant difference between the total mean ELISA in Egypt and Lille ($p < 0.025$).

Table III shows the relationship of the mean values of the data and the mean values of the standard deviations.

Group	Mean		Standard Deviation		Mean		Standard Deviation	
	N	(Mean)	N	(Mean)	N	(Mean)	N	(Mean)
Group 1	10	0.000	10	0.000	10	0.000	10	0.000
Group 2	10	0.000	10	0.000	10	0.000	10	0.000
Group 3	10	0.000	10	0.000	10	0.000	10	0.000
Group 4	10	0.000	10	0.000	10	0.000	10	0.000
Group 5	10	0.000	10	0.000	10	0.000	10	0.000
Group 6	10	0.000	10	0.000	10	0.000	10	0.000
Group 7	10	0.000	10	0.000	10	0.000	10	0.000
Group 8	10	0.000	10	0.000	10	0.000	10	0.000
Group 9	10	0.000	10	0.000	10	0.000	10	0.000
Group 10	10	0.000	10	0.000	10	0.000	10	0.000

Table III shows the relationship of the mean values of the data and the mean values of the standard deviations. There were no significant differences between the mean values of the data and the mean values of the standard deviations. There were no significant differences between the mean values of the data and the mean values of the standard deviations.

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Study No. 111 :-

The release by adult schistosomes after infection of immunosuppressive substance has been demonstrated by Szur et al¹ and Dessait et al². They claimed that this substance which inhibits lymphocyte proliferation, has a low mol.wt. (500 - 1000) and is thermostable.

One of the main problems raised is to know the role of this substance in vivo experiments and to understand if this substance could be responsible for the impairment of the immunological response or how much is it involved in experimental Schistosomiasis.

Work has started to investigate this point and this study will be a preparation for the thesis for the degree of master of science for a research student in our labs who is engaged to work for the project.

Material and Methods:-

Isolation and preparation of immunosuppressive substance.

The method used is that described by Dessait et al². The adult schistosomes (10,000) recovered from 40 days infected hamsters were incubated for 3 hours in 10 ml physiological saline. Supernatant of incubation medium was centrifuged for 5 minutes at 6000 r.p.m. then filtered by millipore filter of 0.45 pore size. The filtrate was dialysed against distilled water (10 ml) at 4 °C for 24 hours the dialysate was collected and filtered through 0.22 pore size millipore filter then lyophilized till needed.

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Animals were divided into 4 groups each of 16 animals (inbred Wistar rats) known as groups A,B,C, and D where :-

Group A : is the control group non infected .

Group B : Injected intraperitoneally daily for 3 successive days with 0.4 ml of the reconstituted lyophilized prepared material so as to give 5 fold concentration .

Group C : Infected and infected , here animals are infected with *S.mansoni* cercariae (500) five days later were infected with same material as group B .

Group D : Infected only . In this group animals are only infected with *S.mansoni* at the same time and with the same batch of cercariae as group C .

At various time intervals after infection 3 rats from each group were sacrificed . Spleen cells of each group were pooled in RPMI 1640 media . Then cells were plated on plastic petri-dish for 2 hours to remove the macrophages. The non adherent cells were then incubated in nylon wool Column for 1/2 hour then eluted with the same media . A suspension of these purified cells were adjusted to 5 million cells /ml .

Cultures were prepared in microtiter , flat bottom plates where each well received 100 ul of cell suspension and 100 ul of concavalin A mitogen .

Group C & D were cultured with schistosome antigen (20,40,60 µg/ml).

Immunological stimulation is manifested by increased synthesis of deoxyribonucleic acid (D.N.A) and determined by measuring the incorporated (H^3) thymidine in the reaction medium . Stimulating agents (mitogen) gives an early response on the 3rd day of incubation while antigen response is usually observed on the 5th day respectively .

Each culture well was pulsed with 0.5 Uci (H^3 thymidine) , 12 hours before termination of culture .

The samples were counted in liquid Scintillation B counter and rate of incorporation is expressed as counts/minute / culture (c.p.m) .

Work is in progress and results will be statistically investigated .

- 1 - Camus ,D ., Dessaint,J.P., Fischer,E. and Capron , A., 1979 . Immunomodulating substances from parasites their role in the regulation of the Immune response . (In Press) .
- 2 - Dessaint ,J.P. , Camus , D., Fischer,E., and Capron, A., 1977 . Inhibition of lymphocyte proliferation by factors produced by schistosoma mansoni .Eur. J. Immunology , 7 : 624 - 629 .

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